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Immunocytochemical Evidence for Production of Luteinizing Hormone and Follicle-Stimulating Hormone in Separate Cells in the Bovine

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ABSTRACT

In all mammalian females, follicular growth and maturation are essentially dependent on the pituitary gonadotropins, FSH and LH. These glycoprotein hormones have many similarities, but their actions, based on high affinity binding to specific membrane receptors, are quite different. The purpose of this study was to perform a sensitive localization of FSH and LH in secretory granules of gonadotrophs using highly specific antisera. This morphological study included light microscopy (PAP) and electron microscopy (immunogold single and double labeling) procedures.

Histologically, approximatively 11.5% of cells were positive for LH, whereas only 5.4% of cells were positive for FSH. With the electron microscope, single labeling allowed identification of morphologically distinct LH-containing cells and FSH-containing cells. Double immunostaining confirmed that no cells contained both hormones. The finding that FSH and LH are produced in separate pituitary cells is in agreement with recent studies that have suggested a specific role and regulatory process for gonadotropins in the bovine species.

INTRODUCTION

The cellular localization of gonadotropins in the pituitary gland remains a controversial matter despite the numerous studies performed during the last two decades. In the early 1970s, the concept of "one cell-one hormone" was widely acknowledged in human as well as nonhuman animals [1–13]. This view was partially challenged when immunocytochemical investigations in various species suggested the presence of a single gonadotroph cell type containing both LH and FSH [14–23].

Some authors, however, described three gonadotropin-containing cell types: specific LH-containing cells, specific FSH-containing cells, and a third bihormonal cell type [24–30]. Childs et al. [31] considered that the percentage of bihormonal gonadotrophs in the rat varies according to the physiological state, and suggested that most gonadotropin-containing cells possess the capacity to produce and store both hormones but perform these functions in separate areas of the same cell. Liu et al. [28], using specific cDNA probes for bovine LH (bLH) and bovine FSH (bFSH) and in situ hybridization in pig pituitary, recently described single hormone (LH or FSH) and double hormone (LH and FSH) gene-expressing cells.

Few studies have been devoted to bovine gonadotrophs: Mikami [7], studying bovine pituitary at light microscope and electron microscope levels, described cytological changes after castration. In line with the "one cell-one hormone concept," he hypothesized the existence of two gonadotroph cell types and presented a morphological description consistent with the findings of the current study. Using immunohistochemical techniques, Dacheux et al. [32]—by electron microscopy—and Girod et al. [33] by immunofluorescence—recognized LH-containing cells but were not able to identify FSH-containing cells in the bovine pituitary.

The interpretation of immunocytochemical techniques for gonadotropins may be difficult because highly specific antisera, yielding no cross-reaction, are not readily available. The primary structure of FSH, thyroid-stimulating hormone (TSH), and LH exhibits many similarities in amino acid sequence and carboxylate content, including a common α subunit. Specific antisera against the β subunit were not suitable for the localization of the native hormone, particularly for FSH. This led many authors to avoid giving definitive value to their observations. Because of these ambiguities, it was decided to revise this subject. Our project was to recognize and localize accurately LH- and FSH-containing cells in the bovine pituitary using highly specific antisera and to establish whether gonadotropins are contained in a single or in two distinct populations of cells.

Our immunocytochemical observations demonstrate for the first time that LH and FSH are produced by distinct cells in the bovine species. The FSH-containing cell was identified and is described in this article.

MATERIALS AND METHODS

Materials

Pituitaries were removed immediately after slaughter from 2 cows and 2 bulls. Both males were 2 yr old (case 1 and case 2). One female was 4 yr old (case 3); the other female was 2.3 yr old and was slaughtered 2 wk after first delivery (case 4).

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Methods

Preparation of antisera: Anti-LH antisera raised in rabbit. Bovine LH was purified until homogeneity was achieved as previously described [34]. Ten rabbits were immunized by intradermal injection of 200 μg of pure LH at 2-wk intervals according to Vaitukaitis et al. [35]. Antisera were extensively characterized for titer, sensitivity, and specificity by RIA as described previously [36]. Using the cross-reaction between LH of rat, sheep, pig, and cow, we measured the bLH with labeled rat LH and ovine LH antiserum. This preceding radioimmunoassay appeared to be specific. No reaction was observed with FSH [37].

Anti-FSH antisera raised in guinea pig. Bovine FSH was purified until homogeneity was achieved by using the method developed in our laboratory [38]. Ten rabbits and 4 guinea pigs were immunized according to the intradermal injection method, with the difference that guinea pigs received incomplete Freund's adjuvant to avoid necrotic lesions and emaciation. Antisera were studied for titer by RIA; rabbit antisera presented lower titer than did guinea pig antisera. Therefore, guinea pig antisera were characterized for sensitivity and specificity. The most sensitive (80 pg/tube) antiserum was used at a final dilution of 1:400 000. The system, however, lacked specificity: LH and α subunit presented a restricted (10%) inhibition of binding from low doses (200 pg). This low inhibition of binding was not modified if the dose of antigen was increased (up to 1 μg). This observation was compatible with the existence of a restricted population of immunoglobulins recognizing the α subunit of the pituitary glycoproteins together with the native FSH. The anti-FSH antiserum was purified by affinity chromatography. Two milligrams of pure α subunit were covalently bound with cyanogen bromide-activated 6B sepharose according to Axen et al. [39]. Two microliters of crude antiserum were chromatographed with a flow rate of 100 μl/h. The purified antiserum was tested with highly purified preparations of bovine LH, TSH, prolactin (PRL; NIH-B4), growth hormone (GH; NIH-B18) and α subunit. No detectable inhibition was observed with any preparation tested up to 1 μg/tube.

Light Microscope Immunocytochemical Procedure

Specimens of pituitary were cut into 5-mm-thick pieces and placed in Bouin's solution. Four- to six-μm-thick sections were used for application of peroxidase-antiperoxidase complex. Sections were deparaffinized with xylene and then rehydrated. Endogenous peroxidase activity was inhibited by incubation in a methanol-H_2O_2 mixture. Nonspecific binding sites for immunoglobulins were blocked by incubation in swine normal serum for 30 min. For antisera dilutions, we used 0.05 M Tris at pH 7.2.

The usual peroxidase-antiperoxidase (PAP) method [40] was used as previously described [41]. Sections were first incubated for 17 h at 4°C with the anti-LH or the anti-FSH antiserum. Working dilutions were 1:100 for anti-LH and 1:100 for anti-FSH. Sections treated by anti-LH antiserum were then incubated at room temperature with swine anti-rabbit immunoglobulins (Dako, G. Postrup, Denmark; dilution 1:20 for 30 min), and thereafter with PAP (Dako, dilution 1:20 for 30 min). Sections treated by anti-FSH antiserum were incubated at room temperature with peroxidase conjugate antibody developed in rabbit (Sigma, St. Louis, MO; dilution 1:20 for 30 min). Peroxidase activity was visualized by incubating sections with 0.01% H_2O_2 and 0.5% diaminobenzidine tetrachlorhydrate in 0.05 M Tris at pH 7.2 for 10 min. Sections were then counterstained with hematoxylin. Controls were performed by incubating sections with normal rabbit serum or with normal guinea pig serum in place of specific antibody.

Electron Microscope Immunocytochemical Procedure

Specimens of pituitary were fixed in 4% glutaraldehyde (in PBS) at 4°C for 2 h. Post-fixation with osmium was omitted. Tissues were rinsed with distilled water, dehydrated in graded isopropylic alcohol and propylene dioxide, and embedded in Epon. Ultrathin sections were picked up on gold grids.

Single immunostaining. The grid-mounted sections were subjected to similar procedures as for human pituitary adenoma [42]. Sections were first treated with 2% normal goat serum (NGS) in PBS for 1 h at room temperature. They were then incubated with the primary antiserum diluted using 2% NGS in PBS (dilution 1:400 for anti-LH and 1:500 for anti-FSH) for 4 days at room temperature in a moist chamber. The grids were afterwards washed with PBS and placed into droplets of PBS for 15 min (twice), and then incubated with the second antibody (gold-labeled goat anti-rabbit or goat anti-guinea pig) for 1 h (dilution 1:20). They were then washed with PBS and rinsed into droplets of PBS for 5 min (twice), and finally washed with distilled water. Staining with uranyl acetate was performed afterwards.

Double immunostaining [42]. Both primary antisera (rabbit anti-LH and guinea pig anti-FSH) were mixed together (dilution 1:400 for anti-LH and 1:500 for anti-FSH) as well as both immunogolds (dilution 1:40). After immunocytochemical procedure, the grids were stained with uranyl acetate and observed with a Philips 300 electron microscope. Sections were observed at magnifications from 1 200× to 70 000×.

Immunogold

Immunogold reagents raised in goats were provided by Janssen Research Pharmaceuticals, Beerse, Belgium. We used goat anti-rabbit antibodies linked to 15-nm diameter gold particles and goat anti-guinea pig antibodies linked to 10-nm diameter gold particles, as described previously [43]. No cross-reaction between immunogolds at working dilution was observed.

TABLE 1. Counts of LH- and FSH-containing cells (mean %).

	LH				FSH			
Subject	Field 1	Field 2	Field 3	Mean	Field 1	Field 2	Field 3	Mean
Male 1	13.15	11.21	10.53	11.63*	8.13	3.63	—	5.81*
Male 2	7.65	15.25	13.92	12.34*	5.63	3.82	2.74	4.09*
Female 1	11.17	12.75	12.76	12.22*	5.55	5.66	5.31	5.51*
Female 2	10.38	11.97	11.79	11.38*	2.57	3.37	5.58	3.82*

*Means are significantly different ($p < 0.06$).

Controls

The specificity of the immunostaining was tested by 1) substituting normal rabbit or normal guinea pig serum for antisera; 2) absorbing each antiserum with its respective antigen; 3) omitting one component of the reaction; and 4) cross-antigen absorption was performed for LH with FSH and for FSH with LH.

Quantification of Immunoreactive Cells

Each slice was examined under a light microscope at a magnification of 400× using a calibrated ocular reticle. The number of pituitary cells positive for FSH or for LH was determined by counting nucleated immunostained cells in three fields of the same slice. A nucleus count of all cells (stained and unstained cells) was also realized in the studied fields. The ratio of immunoreactive cells was determined by dividing the nucleus count of immunostained cells with the nucleus count of all cells.

Stastistical analysis of these counts was also performed using Wilcoxon test after arcsin transformation of the data. We compared LH with FSH for each sex, and male with female for each hormone.

RESULTS

Light Microscopy Investigations

The bovine pituitary is divided into three parts: the pars anterior (PA), the pars intermedia (PI), and the pars nervosa (PN). The residual lumen of Rathke's pouch is located between PI and PA [5]. All our samples were from the PA.

Approximatively 11.5% of cells were positive for LH with no statistical difference between male and female (Table 1). LH-containing cells were scattered or grouped in very small islets uniformly distributed throughout the gland. They usually appeared to be oval or round (Fig. 1a).

Few cells (5.4%) appeared to be positive for FSH without showing a difference between sexes (Table 1). There was a tendency for this percentage to be different from that for LH-containing cells ($p < 0.06$), for each sex, and for the global population. FSH-containing cells often appeared to be scattered and rarely were grouped in small islets. Like the LH-containing cells, they were uniformly distributed throughout the gland. They were oval, triangular, or polygonal (Fig. 1b).

Electron Microscopy Investigations

Both antisera produced confident labeling with very low background. LH cells were of medium size, round or oval in shape, and contained an eccentrically located large nucleus (N) with a prominent nucleolus (Fig. 2a). Most of their small granules (sg), 150–400 nm in diameter, were electron-dense and regularly spherical (Fig. 2, a and b). Accumulation of granules in the periphery of the cell was not uncommon (Fig. 2a). Much larger granules (lg), irregular in shape and less dense than the smallest granules (sg) but more strongly stained, were observed in both sexes (Fig. 2 a and b). That type of very irregular granule was observed exclusively in LH-containing cells. Very little or no staining was found in cytoplasm, mitochondria (m), or the nucleus (N) (Fig. 2a). The secretory granules of adjacent cells were completely devoid of immunostaining (Fig. 2a).

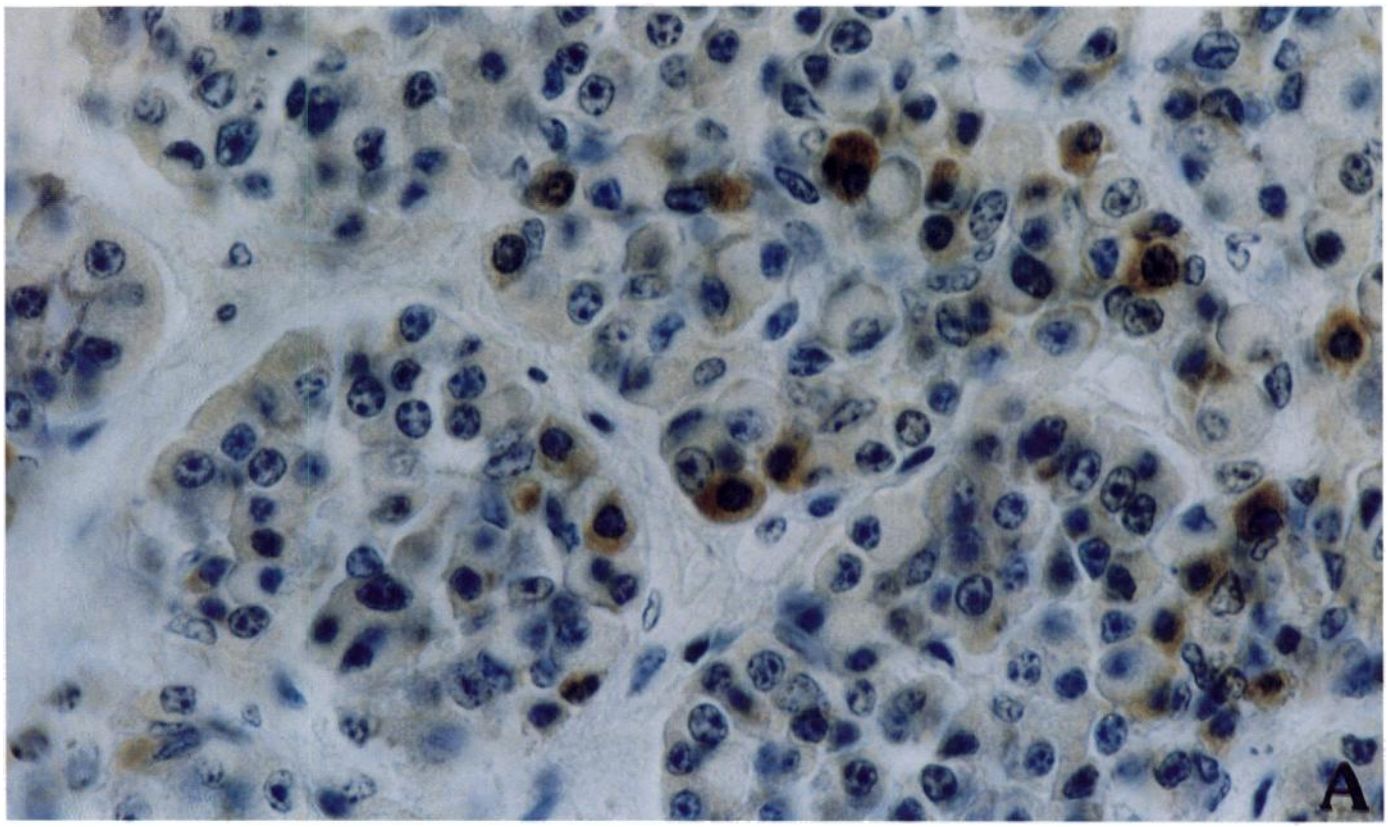
Immunostaining with anti-FSH antiserum resulted in the identification of another cell type. FSH-containing cells were irregular, often triangular or polygonal (Fig. 2c). Granules (g) were electron-dense and rather small (50–200 nm) (Fig. 2c). Some granules, however, appeared to be a little larger and irregular in form—such as elongated and rod- or heart-shaped (Fig. 2, c and d). Mitochondria (m) were usually rod-shaped or elongated (Fig. 2c).

Double immunostaining was also performed in each animal pituitary. In all examined slices, LH-positive and FSH-positive granules were clearly located in distinct cells (Fig. 3). There were no cells that contained both hormones.

DISCUSSION

Despite numerous studies in various species, the existence of unique or heterogenous population of gonadotropin-containing cells remains a controversial matter. At present, very few studies have been devoted to bovine gonadotrophs. Clinical studies in bovine species have suggested specific roles for FSH and LH in follicular growth

FIG. 1. Immunohistochemical localization of LH (Panel A) and FSH (Panel B) in sections of bovine pituitary demonstrating the uneven distribution of both cell types. This figure illustrates the differences in shape of the labeled cells (Panel A: LH cells, round or oval; Panel B: FSH cells, more irregular, often polygonal). Peroxidase anti-peroxidase complex technique. ×250.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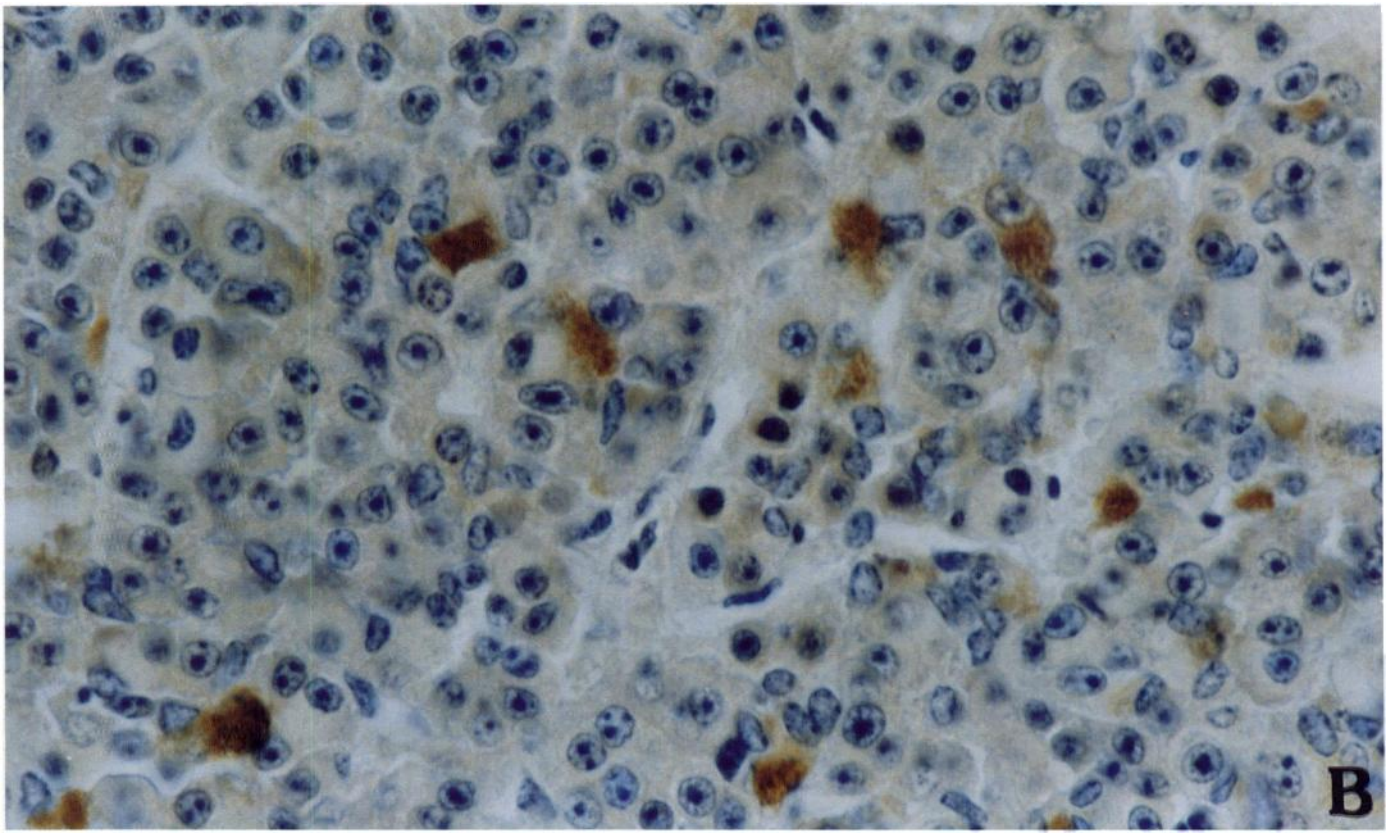
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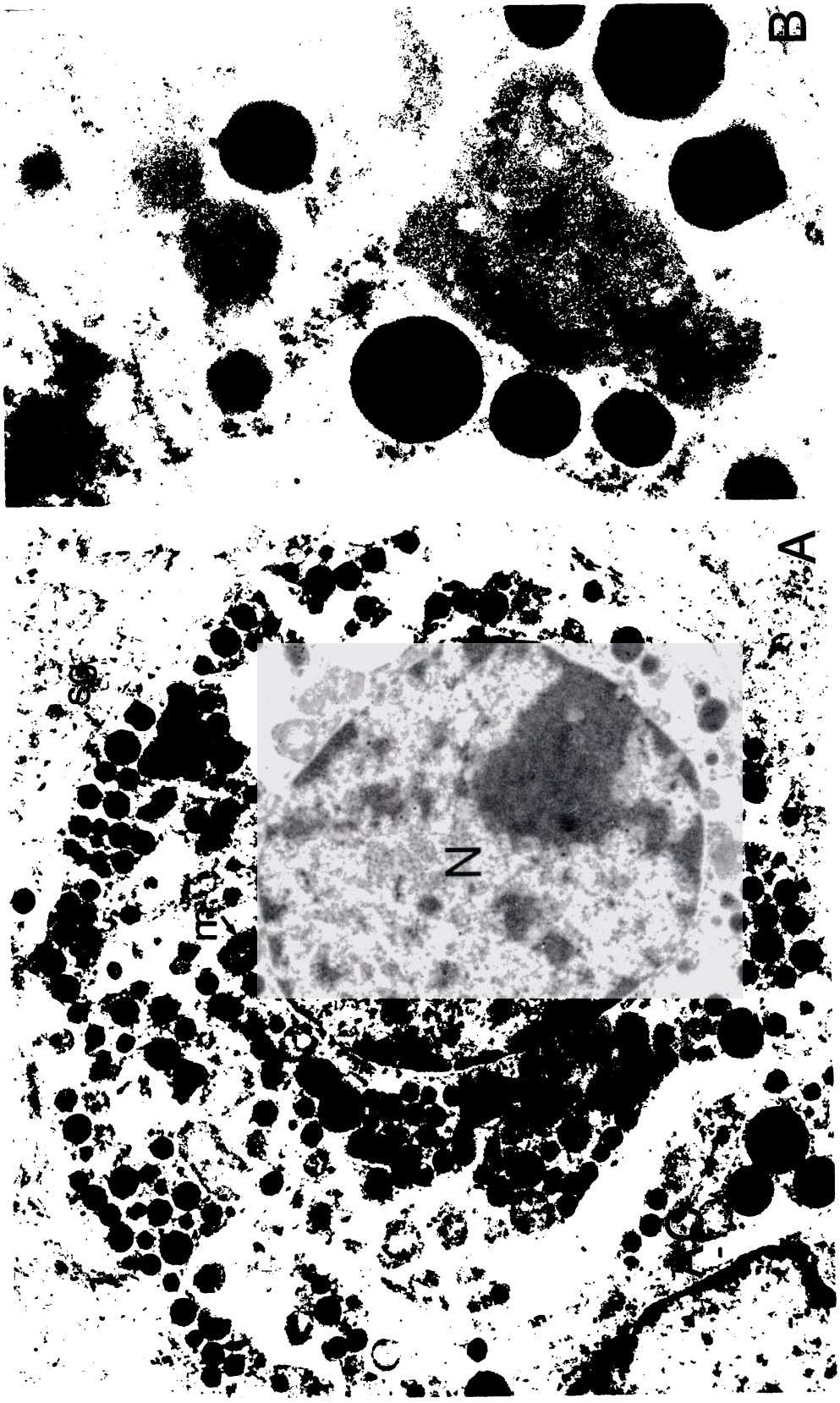
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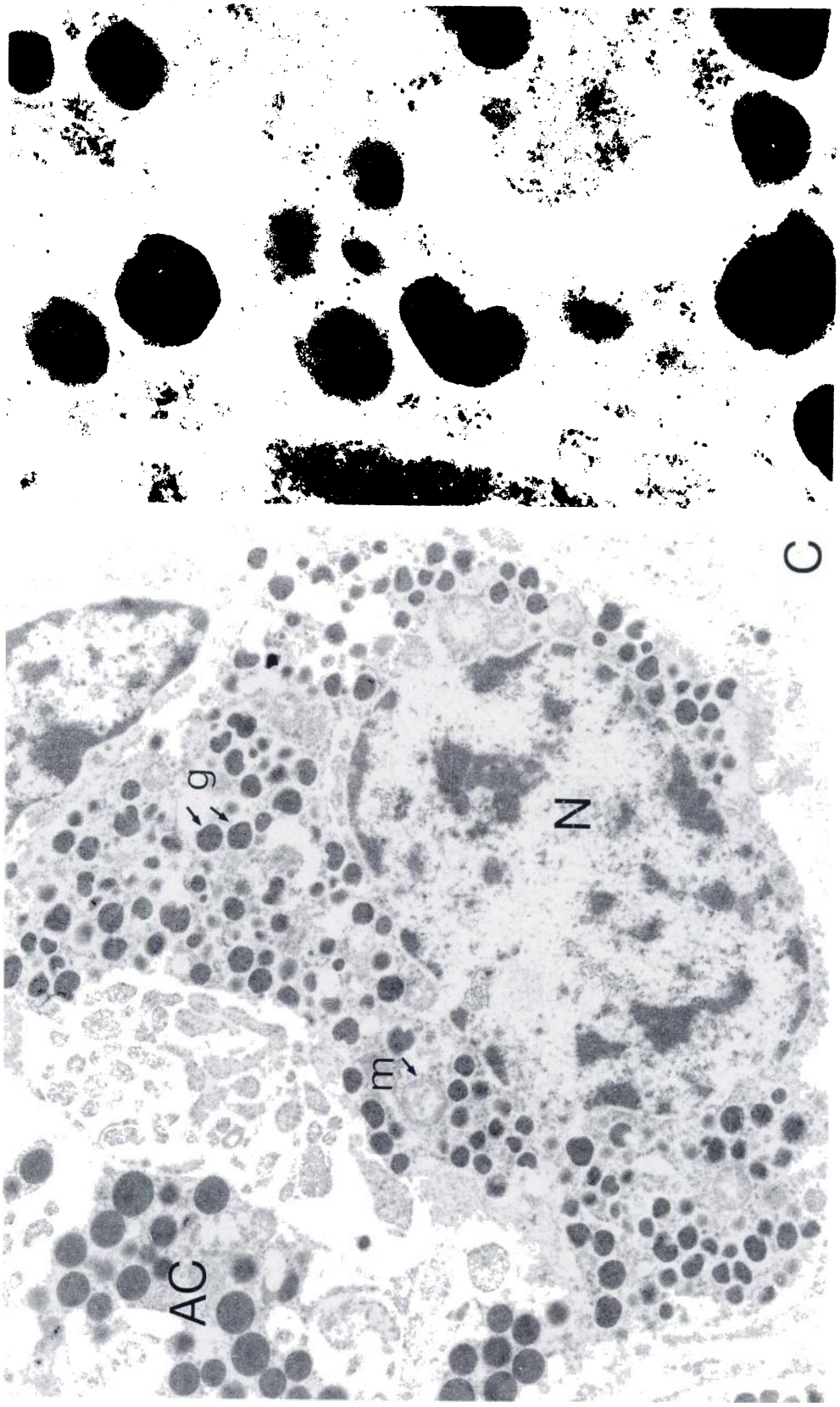

FIG. 2. Panel A) Immunogold electron microscope localization of LH in bovine pituitary cells. The gold particles (15 nm in diameter) can be seen in large irregular granules (lg) and in the more numerous smaller spherical granules (sg). Mitochondria (m) and nucleus (N) are completely devoid of immunostaining, as is the adjacent cell (AC). ×15 096. Panel B) Detail of panel A. ×54 600. Panel C) Immunogold electron microscope localization of FSH in bovine pituitary cells. The gold particles (10 nm in diameter) are exclusively localized in irregularly shaped triangular or polygonal granules (g). Mitochondria (m), nucleus (N), and adjacent cell (AC) are devoid of immunostaining. ×15 096. Panel D) Detail of panel C. ×54 600.

[44, 45]. FSH is responsible for early follicular selection and for increasing the number of healthy follicles, while LH induces atresia during this period [45].

In view of these physiologic observations, we decided to reinitiate a study of LH and FSH localization in bovine pituitary tissue using a sensitive electron microscopy method. The specificity of our antibodies was assessed by RIA. The specific labeling allowed identification of two distinct populations of gonadotrophs. Moreover, these cells could be recognized by their morphological appearance. No cells contained both hormones.

Our data confirm the results of Dacheux [32] who localized LH-containing cells, although she was not able to identify FSH-containing cells in bovine pituitary and could not differentiate between one or two types of gonadotrophs. Mikami [7], studying bovine pituitary at light microscope and electron microscope levels, described cytological changes after castration or thyroidectomy as a clue for the identification of six different types of secretory cells. He interestingly hypothesized the existence of two gonadotroph cell types. He described LH gonadotrophs as medium-sized cells, round or oval in shape, with electron-dense

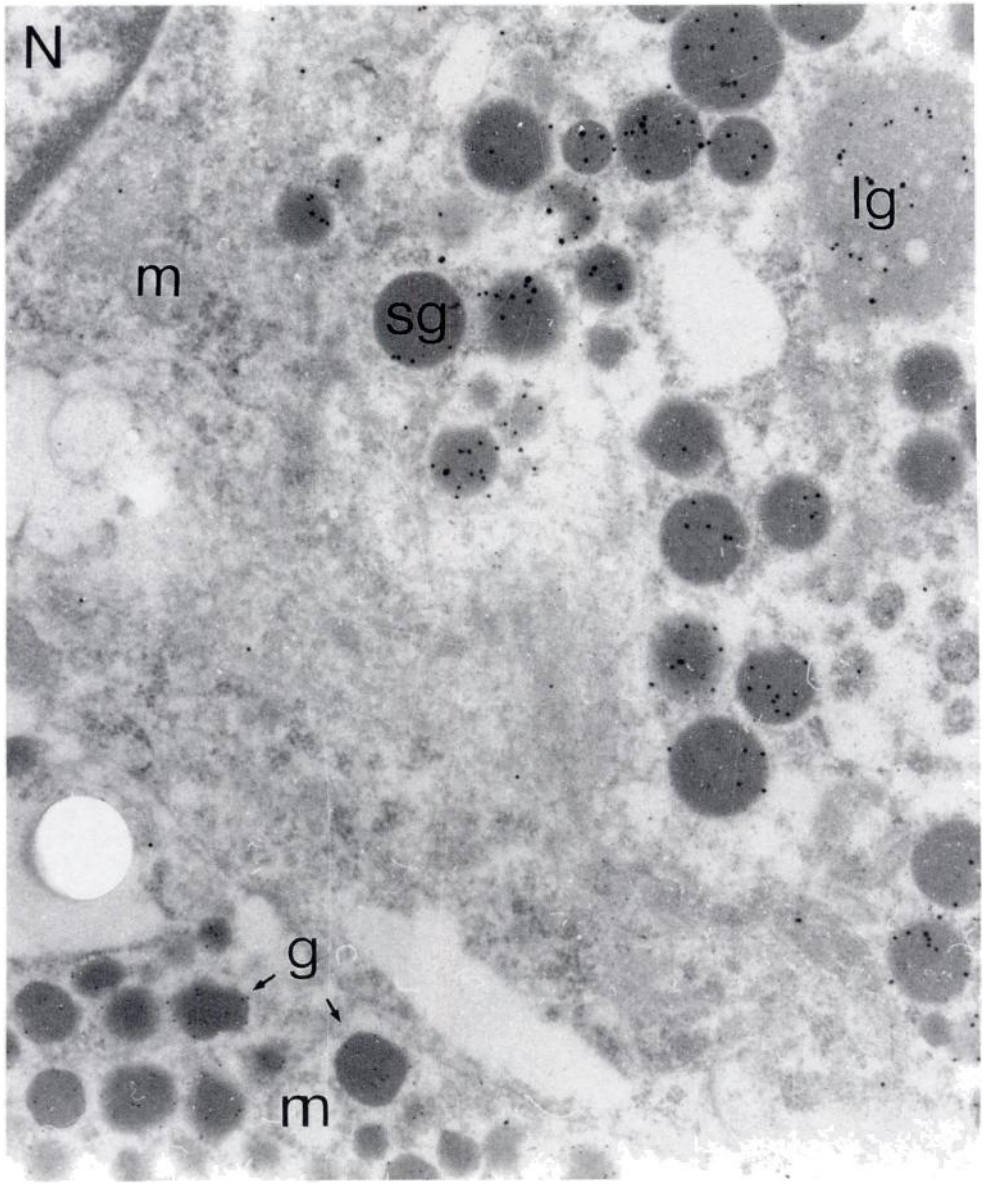

FIG. 3. Double immunogold staining for LH (15 nm) and FSH (10 nm) in bovine pituitary cells. Upper part: A nucleated (N) cell containing both small, dense granules (sg) and large, light granules (lg) labeled with 15-nm particles; recognized as an LH cell. Lower part: A cytoplasmic portion of another cell containing only dense granules (g)labeled with 10-nm particles; recognized as an FSH cell. Mitochondria (m) and nucleus (N) are completely devoid of immunostaining. ×36 450.

and regularly spherical granules, ranging in size from 250–300 μm in diameter. These cells contained dilated vacuoles randomly scattered among the secretory granules. This description is consistent with our findings in LH-containing cells. FSH gonadotrophs were described by Mikami as large in size, oval or polygonal in shape, and containing moderately dense and rather small granules (220–250 μm in diameter). These granules sometimes appeared to be larger and irregular in forms, such as rod- or gourd-shaped. We recognized most of these characteristics in our FSH-containing cells.

Our observations, which favor the concept of two separate populations of gonadotrophs, contradict results obtained by immunocytochemical techniques during the last twenty years in various nonhuman animal species as well as in humans. Indeed most reports mention the existence of cells containing only one type of gonadotropin plus a preponderant gonadotroph containing both hormone cell types. It should, however, be remembered that immunocytochemistry techniques require the use of highly specific antisera yielding no cross-reaction. Such antibodies are difficult to obtain for the study of gonadotroph glycoprotein hormones because of a very high level of homology. Consequently, most previous researchers did not definitively rule out the possibility of a cross-reaction between the two antisera. This technical problem could explain the ambiguity of reported results.

In conclusion, our results constitute the first immunocytochemical evidence that in the bovine species LH and FSH are produced by two separate and morphologically distinct gonadotrophs, with no cells containing both hormones. According to these observations the bovine FSH cell can now be recognized by morphological criteria.

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